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Raimund Nagel
Iowa State University

Axel Schmidt
Max Planck Institute for Chemical Ecology

Reuben J. Peters
Iowa State University, rjpeters@iastate.edu

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Abstract

Main Conclusion

This review summarizes the recent developments in the study of isoprenyl diphosphate synthases with an emphasis on analytical techniques, product length determination, and the physiological consequences of manipulating expression *in planta*.

The highly diverse structures of all terpenes are synthesized from the five carbon precursors dimethylallyl diphosphate and a varying number of isopentenyl diphosphate units through 1'-4 alkylation reactions. These elongation reactions are catalyzed by isoprenyl diphosphate synthases (IDS). IDS are classified depending on the configuration of the ensuing double bond as *trans*- and *cis*-IDS. In addition, IDS are further stratified by the length of their prenyl diphosphate product. This review discusses analytical techniques for the determination of product length and the factors that control product length, with an emphasis on alternative mechanisms. With recent advances in analytics, multiple IDS of *Arabidopsis thaliana* have been recently reinvestigated and demonstrated to yield products of different lengths than originally reported, which is summarized here. As IDS dictate prenyl diphosphate length and thereby which class of terpenes is ultimately produced, another focus of this review is the impact that altering IDS expression has on terpenoid natural product accumulation. Finally, recent findings regarding the ability of a few IDS to not catalyze 1'-4 alkylation reactions, but instead produce irregular products, with unusual connectivity, or act as terpene synthases, are also discussed.

Keywords

Isoprenyl diphosphate synthases, terpenes, isoprenoids, prenyl diphosphates, Arabidopsis

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Isoprenyl diphosphates synthases, the chain length determining step in terpene biosynthesis

Raimund Nagel^{1*}, Axel Schmidt², Reuben J. Peters¹

¹Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA, 50011, USA.

²Max Planck Institute for Chemical Ecology, Department of Biochemistry, Hans-Knoell-Str. 8, D-07745 Jena, Germany

Corresponding Author: Raimund Nagel

rnagel@iastate.edu

Abstract

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The highly diverse structures of all terpenes are synthesized from the 5 carbon precursors dimethylallyl diphosphate and a varying number of isopentenyl diphosphate units through 1'-4 alkylation reactions. These elongation reactions are catalyzed by isoprenyl diphosphate synthases (IDS). IDS are classified depending on the configuration of the ensuing double bond as *trans*- and *cis*-IDS. Additionally IDS are further stratified by the length of their prenyl diphosphate product. This review discusses analytical techniques for determination of product length and the factors that control product length, with an emphasis on alternative mechanisms. With recent advances in analytics, multiple IDS of *Arabidopsis thaliana* have been recently reinvestigated and demonstrated to yield products of different lengths than originally reported, which is summarized here. As IDS dictate prenyl diphosphate length and thereby which class of terpenes are ultimately produced, another focus of this review is the impact that altering IDS expression has on terpenoid natural product accumulation. Finally, recent findings regarding the ability of a few IDS to not catalyze 1'-4 alkylation reactions, but instead produce irregular products, with unusual connectivity, or act as terpene synthases, is also discussed.

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Introduction

Terpenes are a structurally diverse class of metabolites, with currently over 60,000 known compounds (Köksal et al. 2011). Despite this large diversity in structures, they follow the so called 'isoprene rule', that all of them are synthesized from C₅ isopentenoid building blocks (Christianson 2008). They are ubiquitously present in every living organism and despite their broad structural and functional diversity, the initial steps of terpene biosynthesis are highly conserved. The basic C₅ building blocks, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), are synthesized by two pathways, the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways. The MVA pathway uses three units of acetyl-CoA, whereas the MEP pathway starts from glyceraldehyde 3-phosphate and pyruvate (Arigoni et al. 1997; Rodríguez-Concepción 2006). In both pathways, specific regulatory enzymes have been identified (Hemmerlin et al. 2012). The MVA pathway is common to all eukaryotes and some bacteria, whereas the MEP pathway is present in most bacteria and photosynthetic eukaryotes (Lombard and Moreira 2011; Hemmerlin et al. 2012). Plants are distinct as both pathways are present in all species, with the MVA pathway localized in the cytosol and the MEP pathway in the plastids. The MVA pathway produces exclusively IDP, later isomerized to DMADP by isopentenyl diphosphate isomerase (IDI). The MEP pathway produces both DMADP and IDP in a fixed ratio of 1:5-6 (Hemmerlin et al. 2012), which can be altered by IDI for the varying requirements of the organism (Berthelot et al. 2012).

After formation of the basic C₅ building blocks, the smallest terpene with only 5 carbon atoms, isoprene, can be produced directly from DMADP (Behnke et al. 2007). For the biosynthesis of terpenes larger than C₅, isoprenyl diphosphate synthases (IDS) are critical. IDS, also called **prenyltransferases**, catalyze the sequential head-to-tail condensation of IDP with an allylic substrate, usually DMADP, but also geranyl diphosphate (GDP), farnesyl diphosphate (FDP), geranylgeranyl diphosphate (GGDP), geranylgeranyl diphosphate (GFDP), and so on, in order of increasing chain length (Figure 1). For IDS two naming conventions exist. In one the IDS is named after the main product – e.g. geranyl diphosphate synthase (GDPS) produce GDP, FDPS produce FDP, GGDPS produce GGDP, etc. The alternative convention is naming all IDS followed by a number in order of discovery, e.g. IDS1, IDS2 and so on. The first naming convention has the advantage of identifying the product with the enzyme name, while the other is better if one IDS has multiple products or the main product was initially misidentified, as the enzyme would not have to be renamed in such cases.

From the carbon-carbon double-bond configuration of the product, IDS can be classified as either *cis*- or *trans*-IDS (Liang et al. 2002). Although carrying out the same reaction, these are evolutionarily and

structurally distinct from each other. However both types of IDS use divalent cations as cofactors for catalysis, in most cases Mg^{2+} (Liang et al. 2002; Aaron and Christianson 2010). Other metal cofactors have been tested but are generally found to result in lower catalytic activities (Tholl et al. 2001; Sen et al. 2007a; Sen et al. 2007b; Sen et al. 2007c; Vandermoten et al. 2009b; Barbar et al. 2013). The catalytic activity of *trans*-IDS depends on the first and second aspartate rich motifs (FARM/SARM). The FARM is found as either DDxxD or DDxxxxD sequences, whereas the SARM is only present as DDxxD. Both motifs together bind three divalent cations (Mg^{2+}) which themselves bind to the diphosphate of the allylic substrate (e.g., DMADP/GDP/FDP), while IDP is coordinated by basic residues such as lysine and arginine (Aaron and Christianson 2010). Mutations of the aspartate in either the FARM or SARM decreases catalytic activity (Liang et al. 2002). Surprisingly, there is a functional bacterial IDS from *Mycobacterium tuberculosis* whose FARM sequence is altered to DRxxD and its SARM sequence is altered to RDxxA (Mann et al. 2011). *Cis*-IDS lack such distinct motifs, instead they utilize aspartate and glutamate residues distributed throughout the enzymatic sequence. These also bind Mg^{2+} and mutations thereof similarly decrease catalytic activity (Liang et al. 2002).

In addition to the classification of *cis*- and *trans*-IDS, the enzymes can be classified by the length of their products, either short (C_{10} - C_{25}), medium (C_{30} - C_{45}) or long-chain ($\geq C_{40}$) prenyl diphosphates. *Trans*-IDS are more often involved in the biosynthesis of short-chain prenyl diphosphates, whereas *cis*-IDS generally produce longer prenyl diphosphates, with natural rubber containing chains up to 5000 C_5 units in length (Liang et al. 2002; Wang and Ohnuma 2000). However a medium/long-chain *trans*-IDS is present in *Arabidopsis thaliana* (Hsieh et al. 2011) and a group of short-chain *cis*-IDS is found in several tomato species (Sallaud et al. 2009; Schilmiller et al. 2009; Akhtar et al. 2013; Zi et al. 2014).

This review will discuss recent developments in analytical techniques, how product length is determined and the physiological consequences of manipulating IDS expression *in planta*. The focus is on plant IDS, however non-plant IDS are used to illustrate specific aspects that have not yet been investigated with plant IDS.

Analytical measurement of prenyl diphosphate products *in vitro* and *in vivo*

A recent publication revealed that of the over 5,800 annotated IDS only 46 have been biochemically characterized in detail (Wallrapp et al. 2013). In the same publication 79 additional IDS were biochemically characterized. However even with this significant increase of IDS with known product specificity, assigning the length of prenyl diphosphate products by means of sequence comparison is still not accurate. Thus, there is still a need for reliable methods to identify the product distribution of individual IDS.

There are established assays for detection of prenyl diphosphates that use radioactively-labeled [^{14}C]-IDP as substrate, generally followed by acid or alkaline hydrolysis. The resulting prenyl alcohols are easily extracted and then analyzed via radio-gas chromatography (radio-GC), radio-high-performance liquid chromatography (radio-HPLC), thin layer chromatography (TLC) or liquid scintillation counting (LSC) (Zhang and Poulter 1993; Cunillera et al. 1996; Martin et al. 2002; Masferrer et al. 2002; Gilg et al. 2005; Gilg et al. 2009; Manzano et al. 2006; Schmidt and Gershenzon 2008, 2007; Schmidt et al. 2010; Chang et al. 2010; Kim et al. 2010)(Table 1). The previously mentioned methods involve radioactive isotopes, which in most cases require special permits and dedicated lab spaces for their use. A less sensitive method without radio-labeled substrates is GC-MS, although this also necessitates a dephosphorylation step (Demissie et al. 2013; Mann et al. 2011; Akhtar et al. 2013; Schillmiller et al. 2009). Such hydrolysis of the diphosphate is rather laborious, time-consuming and frequently imprecise. In addition, cleavage of the diphosphate under highly acidic conditions usually generates more than one reaction product, making interpretation and quantification fairly complicated (Schillmiller et al. 2009). If instead of acidic conditions, phosphatases are used to cleave the diphosphate enzymatically, the pH of the enzyme assay needs to be shifted first from the optimum for IDS, towards that of the utilized phosphatase. Moreover prenyl diphosphates have been shown to be suboptimal substrates for commercially available phosphatases (Kurokawa et al. 1971). Nevertheless, GC based methods are well suited to resolving different structural and double bond isomers of the same prenyl length (Demissie et al. 2013; Schillmiller et al. 2009). Radio-HPLC in theory should be able to resolve structural isomers, this method was however never used for such purposes. In contrast the resolution of TLC is able to distinguish chain length (Akhtar et al. 2013), but is not able to reliably resolve structural isomers of the same length. LSC can neither resolve chain length nor isomers (Table 1).

The most recently developed method uses HPLC-MS/MS for quantification and works without prior dephosphorylation, improving the signal to noise ratio by two orders of magnitude in comparison to a similar radio-HPLC based assay. This technique has so far been used for short-chain products up to GFDP (C_{25}) and can also distinguish between different double bond isomers of the same prenyl diphosphate length, although additional structural isomers – i.e. irregular prenyl diphosphates – have not yet been tested (Nagel et al. 2012; Nagel et al. 2014; Richter et al. 2015; Nagel et al. 2015; Beran et al. 2016). For prenyl diphosphates longer than GFDP, radioactive assays followed by thin layer chromatography are still the method of choice (Wallrapp et al. 2013; Wang et al. 2016), as ionization of these larger molecules is suppressed in mass spectrometers. This effect can already be observed with GGDP when compared to FDP and GDP (Nagel et al. 2012). Tuning the LC-MS/MS for the detection of solanesyl diphosphate (C_{45}) was not successful, as no ions were detected even after increasing the sample concentration by 100 fold

relative to GGDP. While IDP and DMADP can in theory also be detected with this method, they seem to flow through the column with little interaction with the matrix, eluting very early, and are not separated, preventing distinct quantification of these. Future directions for the development of LC-MS(/MS) based methods would be to increase the ability to reliably analyze IDP and DMADP and also the longer prenyl diphosphates, while increases in sensitivity will largely depend on advances in the mass spectrometers themselves.

The HPLC-MS/MS based method was successfully used for quantification of prenyl diphosphates *in vivo*, representing the first such quantification of GDP, FDP and GGDP together *in planta*. The most recent method describing quantification of GGDP alone dates back to 1983, requiring 1.5 kg of etiolated oat seedlings as starting material, followed by 6 purification steps (Benz et al. 1983). Use of the recently developed HPLC-MS/MS method reduces the amount of requisite plant mass 2000 times (i.e., to 0.75 g), and the purification to a single step using mixed mode interaction SPE columns (Nagel et al. 2014). The amount of GGDP detected in oat seedlings was 10 times higher than the amount in *P. glauca* needles. This can be rationalized by the etiolated state of these seedlings, which would need larger quantities of GGDP for the synthesis of chlorophyll and carotenoids to initiate photosynthesis. Quantification of GDP has been performed in several publications using acid hydrolysis, whereby geraniol is formed from GDP and rearranges to linalool, with subsequent quantification by proton transfer reaction mass spectrometry (PTR-MS). Using acid hydrolysis, significantly larger amounts of GDP (200-fold) were reported for spruce (20 pmol mg⁻¹ FW)(Ghirardo et al. 2010; Nogues et al. 2006) compared to using the HPLC-MS/MS based method (0.1 pmol mg⁻¹ FW)(Nagel et al. 2014). This difference is most likely caused by the presence of already formed linalool or the conversion of other terpenes to linalool under the highly acidic conditions, as already discussed by the authors themselves (Ghirardo et al. 2010). Although the extraction protocol for the HPLC-MS/MS based method already incorporated internal standards that are similar to prenyl diphosphates, the most reliable means of quantification would be stable isotope labeled compounds (Stokvis et al. 2005). Fortunately, a procedure for chemical synthesis of hexadeuterated FDP and GGDP was recently published, which would be useful in supplying standards for such more reliable quantification of prenyl diphosphates *in vivo* (Subramanian et al. 2013).

Quantification of GDP, FDP and GGDP allows comparison with the reported concentrations of their direct precursors IDP and DMADP. Prenyl diphosphate amounts in *P. glauca* vary between 0.1-0.5 nmol g⁻¹ FW in needle tissue (Nagel et al. 2014), whereas DMADP can be found in amounts more than 100 fold higher in *Populus x canescens* leaves (21-36 nmol g⁻¹ DW) and in even higher amounts in pine (50-650 nmol g⁻¹ FW) and *P. abies* (100 nmol g⁻¹ FW) needles (Rosenstiel et al. 2002; Behnke et al. 2007; Ghirardo

et al. 2010). Even though only DMADP was quantified, the equilibrium between DMADP and IDP is established by isopentenyl diphosphate isomerases in the plastid and cytosol (Berthelot et al. 2012). Interestingly the equilibrium is different from the initial ratio produced by hydroxymethylbutenyl diphosphate reductase (HDR) in the plastid (Vickers et al. 2014). The overall DMADP:IDP ratio in kudzu (*Pueraria montana*) was determined to be close to 2 in light adapted plants (Zhou et al. 2013). Though different methods and different species were used for quantifying prenyl diphosphates, the available data indicates that the IDS substrates, DMADP and IDP, are present at higher levels than the IDS products, GDP, FDP and GGDP. Therefore, IDS seem to represent an overall limiting step in terpene biosynthesis, which should be investigated in future research through alteration of IDS expression, even beyond that discussed in detail later in this review.

Determination of Product specificity

The product specificity of short-chain *trans*-IDS is to a large extent regulated by the identity of the amino acids on the N-terminal side of the FARM. Most notably the amino acids in the positions falling 4, 5, 8 and 11 residues before the FARM. These form a pocket that can limit the size of the growing prenyl carbon chain. Increasing the size of those amino acids causes steric hindrance and decreases the length of the final prenyl diphosphate product. The converse is also true, which has led to the molecular ruler theory (Nagel et al. 2015; Wang et al. 2016; Oldfield and Lin 2012; Wang and Ohnuma 2000; Liang et al. 2002; Vandermoten et al. 2009a; Vandermoten et al. 2009b). Recently arginine substitution for two aspartates in the FARM and SARM of IDSs from *Mycobacterium tuberculosis* were identified as another factor that can influence chain length to some degree (Nagel et al. 2018). Some findings further indicate the importance of the interface between IDS subunits, which often form dimers, as this is known to be relevant to product specificity (Ogawa et al. 2011; Chang et al. 2013). In particular, beyond the usual formation of homodimers, certain *trans*-IDS can also form heterodimers/tetramers with non-catalytic small subunits, which can have distinct effects on product outcome or stimulate activity without changing product outcome (Tholl et al. 2004; Orlova et al. 2009; Zhou et al. 2017). For long-chain IDS it has been shown that the purified enzyme requires the presence of detergents or phospholipids for effective turnover. These amphiphilic compounds help sequester the hydrophobic prenyl chain as it is extruded from the enzyme into the aqueous assay buffer. However, in addition to increasing enzymatic activity, use of different detergents can lead to variation in the chain length of the final prenyl diphosphate product (Pan et al. 2013).

Some IDS naturally release prenyl diphosphates of different chain lengths, although this has been most frequently reported with long-chain IDS under *in vitro* conditions (Pan et al. 2013; Hsieh et al. 2011;

Wallrapp et al. 2013). Nevertheless, short-chain IDS also can release multiple products, as demonstrated for IDS from aphids and bark beetles that produce GDP and FDP, and for IDS1 from *Picea abies*, which produces GDP and GGDP, but does not release the intermediately formed FDP (Vandermoten et al. 2008; Vandermoten et al. 2009b; Schmidt et al. 2010). The product specificity of some aphid and bark beetle short chain IDS further appears to be modulated by the ratio of IDP and DMADP (Vandermoten et al. 2008; Vandermoten et al. 2009b; Keeling et al. 2013), which also has been demonstrated with long-chain IDS (Wallrapp et al. 2013).

Besides the factors discussed above, the identity of the divalent metal ion co-factor also has recently been shown to affect product chain length. Such influence was first shown for a long-chain IDS, where exchanging Mg^{2+} to Co^{2+} increased the product size by one C_5 unit (Ohnuma et al. 1993). For short-chain IDS it was reported that Mg^{2+} was the preferred co-factor, with use of other metals usually decreasing enzymatic activity, such that alternative divalent metal ions were typically not investigated, even for the ability to change product output (Tholl et al. 2001; Sen et al. 2007a; Sen et al. 2007b; Sen et al. 2007c; Vandermoten et al. 2009b; Barbar et al. 2013). However, it has recently been reported that two insect IDS, from the leaf beetle *Phaedon cochleariae* and the mosquito *Aedes aegypti*, are able to utilize either Mg^{2+} or Co^{2+} as their metal cofactor without reduced enzyme activity. Instead the identity of the divalent metal co-factor seems to determine product chain length, as FDP is produced with Mg^{2+} and GDP with Co^{2+} (Frick et al. 2013; Rivera-Perez et al. 2015). If both are available, Co^{2+} is preferred for at least the IDS from *P. cochleariae*. These two divalent metal ions have differing atomic radii, which might induce changes in the protein structure that influence the size of the active site cavity. Regardless of exact mechanism, metal co-factor specificity also appears to influence IDS product specificity, although it is unknown if similar mechanisms are used in plants to alter the product specificity of IDS or if this is only relevant in insects.

Given all of the factors described above, it is perhaps not surprising that the length of the prenyl diphosphate produced by *trans*-IDS cannot be accurately predicted through bioinformatics approaches (Wallrapp et al. 2013). In addition, regulation of product length for *cis*-IDS is even less well understood, although it has been reported that the number of amino acids found between conserved regions III and IV of *cis*-IDS varies between those producing long- versus short-chain prenyl diphosphates (Akhtar et al. 2013), and that production of C_{10} versus C_{15} by short-chain *cis*-IDS from tomato is governed by a few amino acids in the active site (Kang et al. 2014).

Trans*-IDS in *Arabidopsis thaliana

Most of the genes involved in terpenoid biosynthesis from the model plant *A. thaliana* have been identified (Tholl and Lee 2011), including the multitude of *trans*-IDS genes found in its genome. However, some of them were initially misidentified. Therefore the latest biochemically characterized activities of the *A. thaliana trans*-IDS are summarized here. The originally designated lone GDPS was recently shown to produce medium/long-chain prenyl diphosphates instead and renamed polyprenyl diphosphate synthase PDPS (Hsieh et al. 2011). The two FDPS, each of which has two splicing variants, all produce FDP as their sole product (Cunillera et al. 1996; Closa et al. 2010; Keim et al. 2012). However, major changes in assigned functionality have been reported for those *trans*-IDS originally all termed GGDPS, of which 11 (renamed to AtIDS1-11) are present in the genome (Tholl and Lee 2011). An additional nonfunctional small subunit also is present, originally named GGDPS12 and since renamed AtSSU (Nagel et al. 2015; Wang et al. 2016). IDS1 is a bifunctional enzyme which produces GGDP and GFDP from IDP and DMADP (Nagel et al. 2015). IDS2 and IDS11 both produce GGDP from IDP and DMADP (Nagel et al. 2015; Wang et al. 2016). However, IDS11 interacts with the small subunit AtSSU, which leads to the release of GDP in addition to the subsequently formed GGDP (Chen et al. 2015; Wang and Dixon 2009). IDS3 and IDS4 also produce GGDP, but only from FDP and IDP, as DMADP does not serve as a substrate for these (Wang et al. 2016). IDS5 is an inactive pseudo-gene (Nagel et al. 2015; Wang et al. 2016). IDS6, IDS7, IDS9 and IDS10 produce GFDP as their sole product from IDP and DMADP (Nagel et al. 2015; Wang et al. 2016). Notably, in the genome the genes for these four IDS are clustered with subsequently acting terpene synthases (TPS) – i.e., these TPS produce cyclized sesterterpene skeletons from GFDP (Shao et al. 2017; Huang et al. 2017). Finally, IDS8 produces polyprenyl diphosphates with chain lengths in excess of C₃₀ (Nagel et al. 2015; Wang et al. 2016).

RNAi and knock out of IDS

Limited information is available about knock-down or knock-out of plant IDS. Double knock-out of FDPS1 and FDPS2 in *A. thaliana* led to a lethal developmental arrest at a very early embryonic stage (Closa et al. 2010). In contrast to this, knock-down by viral induced gene silencing (VIGS) of an FDPS in *Nicotiana attenuata* reduced the transcript abundance, FDPS enzyme activity and sterol content, but the plants were still viable (Jassbi et al. 2008). The advantage of knock-down over knock-out approaches becomes evident when the enzyme is involved in primary metabolism like FDPS. In plants, as in most other eukaryotes, FDP and therefore FDPS are needed for *de novo* sterol synthesis. Sterols are essential for regulation of membrane fluidity and permeability, with further processing leading to hormonal steroids, such as the plant brassinosteroids (Schaller 2003), thus a complete knock-out would not

expected to be viable. The remaining activity of the FDPS in knock-down experiments might be sufficient to supply primary metabolism and still yield an observable phenotype without lethal effects. Knock-down of a GGDPS in *N. attenuata*, which was presumably only involved in secondary metabolism, reduced the amount of diterpene glycosides. This impaired defense against the tobacco hornworm, *Manduca sexta*, but had no adverse side effects on general plant viability in the absence of herbivory (Jassbi et al. 2008). Mutations in GGDPS11/IDS11 in *A. thaliana* affect chloroplast development and reduce chlorophyll and carotenoid concentrations, indicating that GGDPS11 is the major IDS involved in production of these compounds, as was also indicated by the finding that this was the hub enzyme in bioinformatics investigations (Ruiz-Sola et al. 2015; Ruppel et al. 2013). Interestingly overexpression of one of the GFDPS in *A. thaliana* also led to a chlorotic phenotype, most likely by depleting the IDP and DMADP pool and/or further conversion of GGDP to GFDP, which can then not be used for chlorophyll and carotenoid biosynthesis (Nagel et al. 2015).

Overexpression of IDS

Overexpression of IDS has only been successfully achieved in plants, as it has not been possible to do this in animals, with the ensuing results depending on the individual IDS and plant species. FDPS1 overexpression causes severe alterations in cytokinin homeostasis and induces senescence in *A. thaliana* (Masferrer et al. 2002; Manzano et al. 2006). By contrast, overexpression of a different FDPS in *Artemisia annua* increases artemisinin levels without the severe effects observed in *A. thaliana*, although some phenotypic variation in leaf morphology were noted (Han et al. 2006; Banyai et al. 2010; Kai et al. 2011). However, artemisinin biosynthesis is somewhat unique, as IDP and DMADP are not exclusively supplied by the MVA pathway as expected for a sesquiterpene. Instead, one IDP unit originates from the MEP pathway (Towler and Weathers 2007; Schramek et al. 2010). Unlike the previously mentioned IDS which are all localized in the cytosol, other IDS are localized to the plastid. Similar to the case in *A. annua*, overexpression of these has successfully targeted secondary (more specialized) metabolism. For example, with the native GGDPS of *Salvia miltiorrhiza* in hairy root cultures, which increased diterpenoid tanshinone levels (Kai et al. 2011), while overexpression of a GDPS in mint increased the total monoterpene content (Lange et al. 2011), and overexpression of a bifunctional IDS producing GDP and GGDP in *Catharanthus roseus* increased secologanin production, which was further enhanced by co-expression of a geraniol synthase (Kumar et al. 2018). Overexpression of the bifunctional GDP/GGDP IDS1 in *P. glauca* increased *in planta* prenyl diphosphates concentrations and the levels of total terpenoids, the later however by production of geranylgeranyl fatty acid ester diversion products (Nagel

et al. 2014). By contrast, IDS overexpression increased levels of the major terpenoids in mint, *C. roseus* and *S. miltiorrhiza*. It appears that overexpression of IDS in plants is strongly dependent on the subcellular compartment and cell type in which the enzyme is expressed and on the proportion of IDP and DMADP supplied by the MVA and MEP pathways. This hypothesis was tested by overexpressing a TPS or a TPS together with an IDS either in the cytosol or plastid of *Nicotiana tabacum* (Wu et al. 2006). The authors chose a monoterpene or sesquiterpene producing TPS together with an IDS thought to produce either GDP or FDP. Overexpression of the IDS producing FDP in combination with a sesquiterpene producing TPS increased the amount of sesquiterpenes compared to overexpression of the TPS alone. Moreover, if both enzymes were localized in the plastid rather than in the cytosol even greater increases were observed, indicating regulatory differences between the two compartments. Unfortunately, for monoterpene analysis these studies utilized the IDS that was then thought to produce GDP as its main product, but that since been shown to actually produce medium/long-chain prenyl diphosphates instead (Hsieh et al. 2011). This then provides a rationale for the reported minimal increase in monoterpenes after combined overexpression, and is consistent with production of longer chain products by this IDS. Accordingly, the effect of IDS overexpression depends on different cellular compartments (Wu et al. 2006), but also on the combination of promoter and investigated tissue. Another similar example arises from use of a fruit ripening specific promoter in tomato with a *cis*-IDS producing neryl diphosphate (NDP). The fruits of these plants showed a major increase in NDP derived monoterpenes coupled to a significant decrease of lycopene. This reduction of lycopene was traced to an inhibitory effect of the *cisoid* NDP on the activity of *trans*-GGDPS (Gutensohn et al. 2014).

Altered expression of non-catalytic small subunits

For some IDS, their activity and/or product specificity are altered upon interaction with non-catalytic small subunits (SSU). There are a few studies that investigate the consequences of terpene metabolism upon alteration of the expression of these SSU *in planta*. For instance overexpression of the *Arabidopsis* SSU slightly increased the amount of monoterpenes emitted by flowers, while SSU T-DNA insertion lines showed a more pronounced decrease of monoterpene emission (Chen et al. 2015). Expression of the SSU from snap dragon (*Antirrhinum majus*) in tobacco with a petal specific promoter from *Clarkia breweri* increased monoterpene emissions, but at the same time significantly reduced the levels of chlorophyll, carotenoids and gibberellins. These plants exhibited leaf chlorosis and a significant dwarfing phenotype. The results indicate that the chosen promoter does not direct petal specific expression in tobacco (Orlova et al. 2009). A more targeted expression of the same SSU in ripening

tomato fruits led to less drastic effects on overall plant physiology and increased monoterpene emissions, albeit with decreased lycopene content, only in fruits (Gutensohn et al. 2013). A special case is the interaction of the SSU from rice (*Oryza sativa*) with its native GGDPS, which does not alter product chain length distribution, but instead increases overall GGDPS activity. Overexpression of this SSU in rice increased chlorophyll content, along with slight reductions in plant height and carotenoid content. RNAi mediated reduction in SSU expression or knock-out mutants exhibited major reductions in carotenoid and chlorophyll content, as well as reduced height (Zhou et al. 2017). Altogether, it appears that alteration of SSU expression can have varying, even drastic effects on plant physiology. The effects strongly depend on the particular SSU (alteration of activity vs. product specificity) and heterologous expression pattern (global vs. tissue specific). Given the small number of publications available, there is need for future research to better understand how SSUs regulate terpene biosynthesis *in planta*.

IDS catalyzing alternate reactions

Some IDS from both the *cis*- and *trans*-IDS families catalyze usual reactions that differ from the usual 1'-4 alkylation reactions between IDP and DMADP, instead carrying out coupling reactions between two molecules of DMADP, or DMADP and another allylic substrate. One *cis*-IDS from *Lavandula x intermedia* produces lavandulyl diphosphate (Demissie et al. 2013) and two IDS from *Streptomyces* spp. produce cyclolavandulyl diphosphate or isosesquilavandulyl diphosphate (Ozaki et al. 2014; Teufel et al. 2014)(Figure 2). Their exact enzymatic mechanism has not yet been investigated in detail. However, mutational analysis of a *cis*-IDS from *Solanum habrochaites* identified the residue at position 103 as important for such irregular coupling, as a H103Y mutant of this produces limonene, lavendulol and an unidentified irregular product in addition to the (Z,Z)-FDP produced by the wild type enzyme (Chan et al. 2017). Crystal structures for cyclolavandulyl diphosphate synthase and isosesquilavandulyl diphosphate synthase, each both alone and in substrate bound complexes, were recently reported (Gao et al. 2018; Malwal et al. 2018). However, the critical amino acids responsible for their deviation from the usual 1'-4 alkylation reactions between IDP and DMADP towards irregular product linkages were not identified.

Trans-IDS from *Chrysanthemum cinerariaefolium* and *Artemisia tridentata* ssp. *spiciformis* have also been shown to couple two molecules of DMADP to produce chrysanthemyl diphosphate via an unusual 1'-2-3 alkylation reaction (Rivera et al. 2001; Hemmerlin et al. 2003)(Figure 2). In both enzymes the ability to form irregular products was investigated via side directed mutagenesis, and alterations in the SARM, which takes the form of NDxxD instead of DDxxD, were identified as critical for this unusual reaction (Erickson and Poulter 2003; Thulasiram et al. 2008). More recently the same IDS from *C.*

cinerariaefolium was further shown to also act as a TPS, producing chrysanthemol from chrysanthemyl diphosphate (Yang et al. 2014).

Similar to these findings, insects are now emerging as a rich source of IDS that carry out atypical reactions. Four *trans*-IDS from the flea beetle *Phyllotreta striolata* and one *trans*-IDS each from the harlequin bug *Murgantia histrionica* or Southern green stink bug *Nezara viridula* were shown not to catalyze prenyl elongation steps at all, but to be bona fide TPS (Beran et al. 2016; Lancaster et al. 2018a; Lancaster et al. 2018b). *P. striolata* also has an unusually high number of IDS and IDS-like enzymes in its genome and another *trans*-IDS was shown to produce the *cisoid* products neryl diphosphate from IDP and DMADP and (Z,E)-FDP from (E)-GDP and IDP, making it the first *trans*-IDS to yield *cisoid* products (Beran et al. 2016)(Figure 2).

Conclusion

IDS mediate an essential branch point in terpene biosynthesis, as they dictate the length of terpenes produced by a cell/organism. It is therefore essential to understand the product profile of individual IDS and the factors influencing their product length. This review summarizes recent developments in analytical techniques for such IDS product analysis, along with investigation of the factors that alter the product profile of *trans*-IDS. For *cis*-IDS our understanding of product outcome is more limited and should be further investigated in the future. Similarly, our understanding of the role of IDS in overall regulation of terpene biosynthesis needs to be advanced as well. Major future directions are tissue and cellular compartment specific alterations of IDS activity, instead of the current use of promoters with a global expression pattern. At the same time shifting the focus from the end products of terpene biosynthesis towards investigating alterations of the pathway intermediates would help to identify bottlenecks within such metabolism and specifically determine if and when IDS represent the bottleneck step in these pathways. During the revision of this review a study reported a specific increase of carotenoids in Arabidopsis through expression of a protein fusion between AtIDS11 and phytoene synthase (Camagna et al. 2018). Similar fusions with other downstream enzymes could be a novel strategy to specifically increase individual terpenes or at least a subset of terpenes. As more IDS, both *trans* and *cis*, are identified that catalyze irregular reactions, previously inactive IDS might need to be revisited and tested for their ability to catalyze similar or additional unusual reactions, which is especially applicable to insects, with at least two such reports appearing this year (2018).

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Figure 1

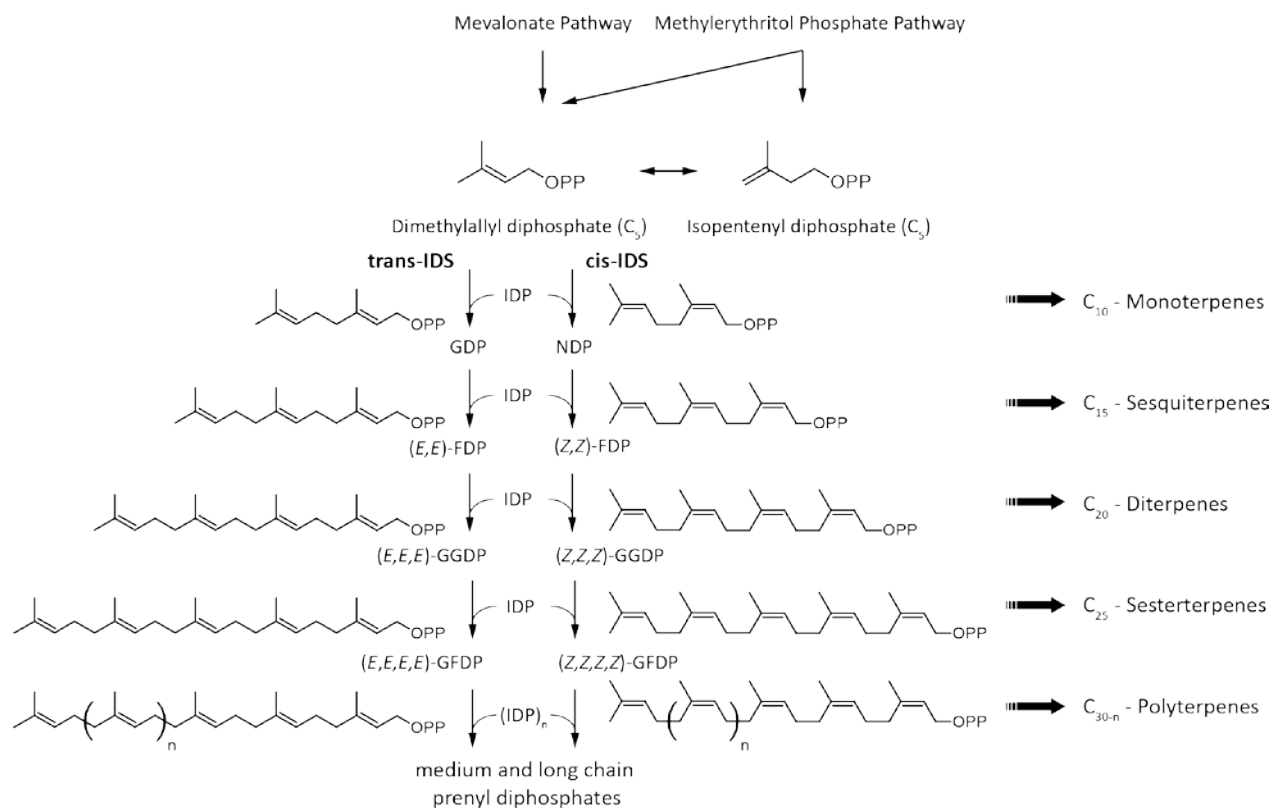


Figure 1: Reactions catalyzed by isoprenyl diphosphates synthases. Dimethylallyl diphosphate (DMADP) and a varying number of isopentenyl diphosphate (IDP) are used as substrates by isoprenyl diphosphate synthases (IDS) to generate longer prenyl diphosphate chains, which are the precursors for the indicated terpene classes. Depending on the stereoisomer of the double bond IDS are classified as either *trans*- or *cis*-IDS. Geranyl diphosphate and neryl diphosphate are the *trans* or *cis*-isomers at the C_{10} stage. Longer prenyl diphosphates share the same name and the double bond isomer is classified by *E* or *Z* nomenclature starting from the double bond closest to the diphosphate.

Figure 2

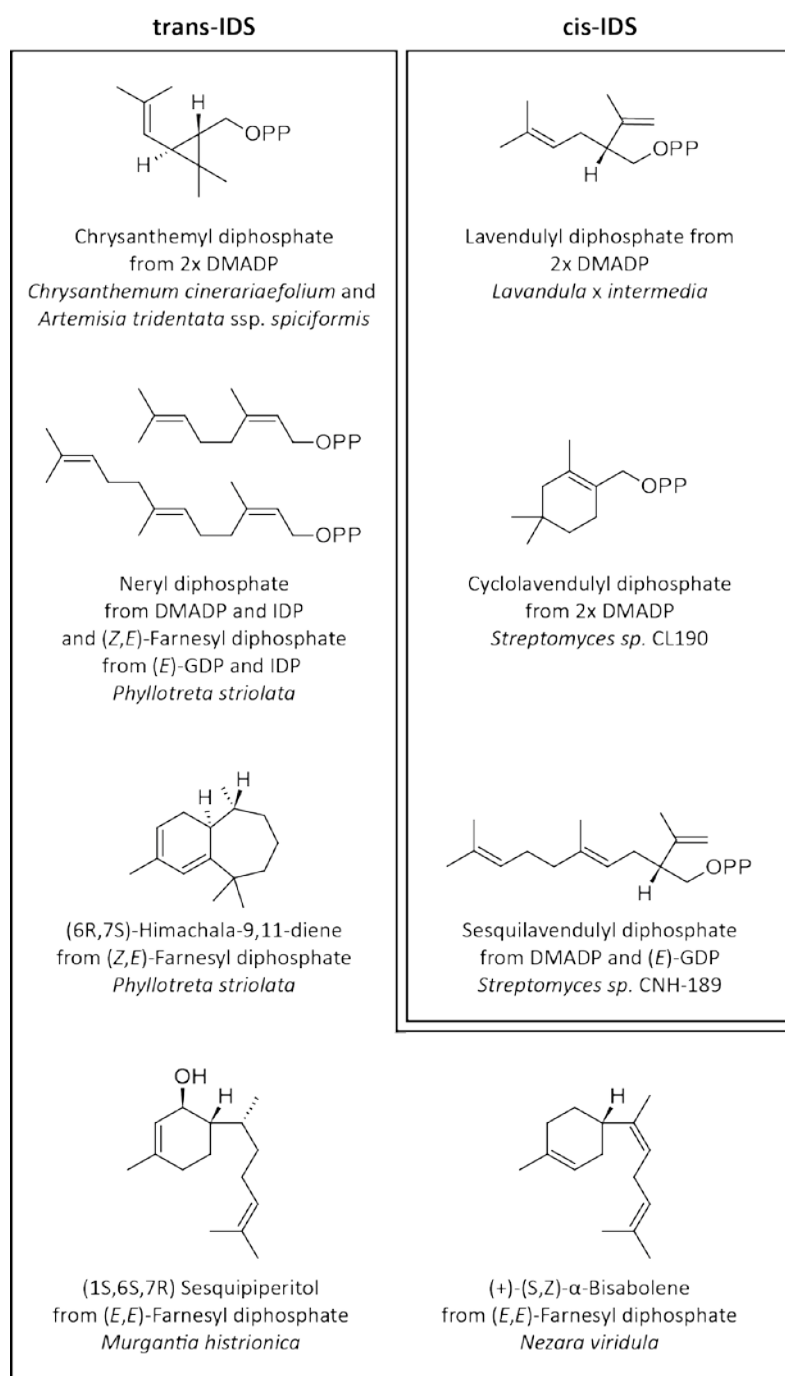


Figure 2: Irregular products produced by IDS. Depiction of products from *trans*- and *cis*-IDS that do not follow the usual 1'-4 alkylation reaction or have unexpected stereochemistry. Besides the structure the organism and the substrates for the product are indicated.